The soluble terminal complement complex (SC5b-9) up-regulates osteoprotegerin expression and release by endothelial cells: implications in rheumatoid arthritis

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Objective. Complement activation products contribute to a large number of inflammatory diseases, including RA. We have investigated whether osteoprotegerin (OPG) may concur with the soluble terminal complement complex (SC5b-9) to the inflammatory cascade characterizing RA.

Methods. Levels of SC5b-9 and OPG in the plasma and SF of patients with active RA were determined by ELISA. The presence of SC5b-9 and OPG in RA synovial lesions was analysed by immunohistochemistry. Cultured endothelial cells were used for *in vitro* leucocyte/endothelial cell adhesion assays. In addition, endothelial cells were exposed to SC5b-9 in order to evaluate the effects on the production of OPG protein, as well as the activation of the OPG promoter.

Results. Patients affected by active RA are characterized by elevated levels of both SC5b-9 and OPG in plasma and/or SF. Of note, we have observed a co-localization of SC5b-9 and OPG in endothelial cells of post-capillary venules of RA synovial lesions. Data on endothelial cell cultures showed that exposure to SC5b-9 induced the up-regulation of OPG expression/release, stimulating the transcriptional activity of the OPG promoter, and synergized with TNF-α in up-regulating OPG production.

Conclusions. Our findings demonstrate that SC5b-9 induces OPG production by endothelial cells and we propose that the SC5b-9-mediated up-regulation of OPG may be an important mechanism whereby complement contributes in promoting and/or enhancing the inflammation in RA.

KEY WORDS: Endothelium, Osteoprotegerin, Inflammation, Complement system.

Introduction

The importance of complement system in the development and amplification of the inflammatory process at the tissue level in various pathological conditions, including RA, has been previously demonstrated [1–3]. The pathogenesis of RA is a multi-step event and, besides the contribution of both cell-mediated and antibody-dependent tissue damage, complement has been recognized as one of the key initial events occurring in the acute phase of RA and it may also be responsible for amplification of the inflammatory process occurring during progression of RA [1–3]. Complement activation products are detected both in the circulation [4–6] and in the SF of patients with RA, and their contribution to damage of the synovial membrane is supported by detection of complement deposits in synovial tissue during the chronic phase of RA [7–10]. Moreover, the beneficial effect of anti-complement therapy has been demonstrated in animal models of arthritis [11–13], which share with human RA the features of an inflammatory synovitis characterized by synovial hyperplasia and leucocyte infiltration and, as in the chronic phase of RA, pannus formation and cartilage/bone erosion. In particular, increasing evidence from our group show that the cytolytically inactive soluble terminal complement complex (SC5b-9) exerts several pro-inflammatory responses acting directly on endothelium, such as the induction of adhesion molecules [14] and the increase of vascular leakage [15].

Osteoprotegerin (OPG) is a soluble member of the TNF receptor super-family that has been originally characterized for its ability to suppress osteoclast formation [16] by binding to RANK ligand (RANKL), and preventing the interaction of RANKL with its high-affinity transmembrane receptor, namely RANK [17]. OPG also interacts with TNF-related apoptosis inducing ligand (TRAIL) [18], a death-inducing ligand whose extracellular domain shares ~30% homology with RANKL and shows the ability to interfere with the osteoclast formation induced by RANKL [19]. Several reports have previously shown increased serum levels of OPG, as well as of RANKL, in patients affected by RA [20–26], but the physiopathological significance of the up-regulation of OPG in RA patients is not well understood. Interestingly, a possible pathogenetic link between elevated levels of OPG and inflammation has been suggested by recent *in vitro* studies of our group [27] and Mangan *et al.* [28] demonstrating that recombinant OPG promotes leucocyte adhesion to endothelial cells.

On this basis, the aim of our study was to investigate potential link between SC5b-9 and OPG in the promotion of the inflammatory cascade characterizing RA, by evaluating the possibility that SC5b-9 might modulate the expression and/or release of OPG in endothelial cells.

Materials and methods

Patients' samples

Plasma and SF were obtained from 10 patients with active RA (44–72 years, three males/seven females) fulfilling the ACR classification criteria [29]. The mean disease activity score was 3.2 and the mean disease duration was 4.5 years. All patients were treated with systemic corticosteroids and, in addition, with leflunomide, or HCQ, or rituximab, or MTX. From each patient, plasma was

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obtained from venous blood, while SF was obtained during joint aspiration from knee joints, and collected in heparinized syringes. Venous blood and SF samples were centrifuged at 370 and 250 g, respectively, for 10 min and the resulting supernatant was stored at $-80°C$, in multiple aliquots, until analysis. As control, plasma was collected from 20 age- and sex-matched healthy donors. Written consent for all procedures was obtained from all subjects in accordance with the Declaration of Helsinki and the institutional review board of the University Hospital of Ferrara gave ethical approval for the study.

ELISA

OPG levels were measured in plasma and SF samples as well as in endothelial cell culture supernatants using a sandwich-type ELISA kit purchased from Alexis Biochemicals (Lausen, Switzerland), as previously described [30]. The presence and the levels of SC5b-9 into the plasma and SF were evaluated by ELISA as previously described [14], while the levels of TNF-α were analysed by using an ELISA kit purchased from R&D Systems (Minneapolis, MN, USA). All measurements were done in duplicate and the results were read at an optical density of 450 nm using an Anthos 2010 ELISA reader (Anthos Labtec Instruments Ges.m.b.H, Salzburg, Austria).

Immunohistochemistry detection of SC5b-9 and OPG

Samples of synovial membranes ($n = 5$) were gathered from the archives of the Department of Human Pathology of the University of Palermo, as approved by the local institutional review board. All specimens were fixed for 24 h with 10% buffered formalin, paraffin-embedded and cut in serial sections of $5 \mu m$. Tissue morphology was evaluated by haematoxylin and eosin staining. Immunohistochemical detection of SC5b-9 and OPG was performed on serial sections by using the neoepitope-specific anti-C9 antibody (clone WU 13-15; kind gift of Prof. R. Wuerzner, Austria), and the anti-human OPG monoclonal antibody (clone 69 146; R&D Systems), respectively. After dewaxing and endogenous peroxidase inhibition, slides were microwave-oven heated three times for 5 min in TRIS/EDTA pH 9.0 buffer (heat-induced epitope retrieval) and then washed with phosphate-buffered saline (PBS). After 30 min incubation with the primary antibodies (diluted in PBS) at 37°C, followed by at least two PBS washings, the Novolink Polymer HRP IHC detection kit (Novocastra, UK) was used for 30 min at room temperature, according to the manufacturer's instructions. Romulin-aminoethylcarbazole was used as a chromogen for 5 min at room temperature with subsequent nuclear counterstaining with haematoxylin. Negative controls (omission of the primary antibody, and use of an irrelevant antibody of the same isotype as the primary antibody) were included in each immunohistochemical labelling run.

Cell cultures and treatments

Human umbilical vein endothelial cells (HUVECs) were purchased from BioWhittaker (Walkersville, MD, USA) and grown on 0.2% gelatin-coated tissue culture plates in M199 endothelial growth medium supplemented with 20% fetal bovine serum, $10 \mu g/ml$ heparin and $50 \mu g/ml$ ECGF (all from BioWhittaker). In all experiments, cells were used between the 3rd and 5th passage *in vitro*, as previously described [31]. EDTA-blood samples for the isolation of primary polymorphonuclear neutrophils (PMNs) were drawn from healthy volunteers after obtaining informed consent. Blood samples were diluted 1:2 with PBS and a first separation step was performed by Histopaque ficoll gradient centrifugation (Lymphoprep; Nycomed, Oslo, Norway). PMNs in the pellet of the gradient were separated from erythrocytes by further steps consisting of dextran sedimentation and hypotonic lysis, resulting in purity >90% CD15/CD11b$^+$ cells, as assessed by flow cytometry using specific FITC- or phicoeritrin (PE)-conjugated

monoclonal antibodies (Immunotech, Marseille, France; Miltenyi Biotech GmbH, Bergish Gladbach, Germany).

For cell treatments, the following reagents have been used: recombinant human OPG and TNF-α (both purchased from R&D Systems), and SC5b-9, prepared and purified as previously described [15].

Endothelial–leucocyte adhesion assay

Vascular endothelial cells were grown to confluence in 24- or 96-well tissue culture plates and stimulated for 16 h at 37°C with SC5b-9 or recombinant OPG. After three washings with serum-free medium, untreated $1–3.5 \times 10^5$ PMN cell suspensions were added to each well and were further incubated at 37°C for 30 min. After endothelial–leucocyte co-culture, non-adherent PMNs were removed by washing the wells at least twice. Endothelial–leucocyte co-cultures were photographed under a light microscope ($10\times$ magnification). The number of adhered PMNs was evaluated by a colorimetric assay using tetramethyl benzidine (Sigma Chemicals, St Louis, CA, USA) as a substrate for myeloperoxidase, as previously described [32], and/or by scoring at least six random fields for each treatment. In the assays, the viability of both endothelial cells and adherent PMNs was routinely monitored at light microscopy by Trypan blue dye exclusion or by flow cytometry after propidium iodide (PI) staining, performed as previously described [33, 34].

Transfection experiments

To generate the OPG-promoter reporter plasmid (pOPG-Luc) a 1172-bp fragment of the OPG gene 5 flanking region was amplified from HUVEC genomic DNA by PCR using the following primers 5'-<u>AGATCT</u>CTGGAGACATATAACTTGAAC ACTTGGCCC-3' and 5'-<u>GAATTC</u>TGTGGTCCCCGGAAACC TCAGG-3' (restriction sites are underlined). This fragment was cloned into *Bgl*II/*Eco*RI sites in the pMetLucReporter Vector (Clontech, Mountain View, CA, USA). Subconfluent HUVECs were detached and resuspended in the specified electroporation buffer to a final concentration of 7×10^5 cells/ml. Two micrograms of luciferase-reporter plasmids (either pOPG-Luc or control vector pMetLuc-Reporter, Clontech) were mixed with 0.1 ml of cell suspension, transferred to electroporation cuvettes and nucleofected with the human HUVEC nucleofector kit (Amaxa, Cologne, Germany) using the program A-034 of the nucleofector device (Amaxa NucleofectorII apparatus). In parallel, cells were transfected using the control EGFP-plasmid (Amaxa) and transfection efficiency was monitored in each experiment by scoring the percentage of fluorescent EGFP-positive cells. After electroporation, cells were immediately transferred to complete M199 medium, cultured in 24-well plates and allowed to recover before treatment with SC5b-9. The secreted luciferase was measured with the ready-to-glow secreted luciferase reporter system (Clontech) according to the manufacturer's instructions at 405 nm in a luminometer, and as previously described [35]. Results were normalized for transfection efficiency, determined in each experiment by co-transfection of a β-galactosidase-control plasmid under the control of a constitutive promoter (SV40) (β-gal Reporter System, Clontech).

Assay for NF-κB DNA binding

NF-κB induction was measured using the Trans-AM NF-κB p65 kit (Active Motif, Rixensart, Belgium), which measures the level of active form of NF-κB contained in cell extracts, able to specifically bind to an oligonucleotide containing the NF-κB consensus site (5'-GGGACTTTCC-3'), attached to a 96-well plate. Assays were performed in duplicate, according to the manufacturer's instructions. NF-κB DNA binding activity was determined as absorbance values measured by using an Anthos 2010

ELISA reader. Increase in fluorescence was linear over extract concentration.

Statistical analysis

The media, median, minimum and maximum values were calculated for each group of data. For each set of experiments, values are reported as means ± S.D. For selected experiments, results are reported as box plots showing the median, minimum and maximum values and 25th to 75th percentiles. Data were analysed by ANOVA and with the Mann–Whitney rank-sum test. Comparison of group means was performed by Bonferroni method. Statistical significance was defined as $P < 0.05$.

Results

SC5b-9 and OPG are elevated in the plasma of patients affected by active RA and co-localize in the endothelium of synovial tissue

In the first set of experiments, we measured the levels of SC5b-9 and OPG in the plasma and SF of patients with active RA. As shown in Fig. 1A, SC5b-9 and OPG plasma levels were significantly ($P < 0.05$) increased in RA patients compared with the healthy controls. Interestingly, however, while the levels of SC5b-9 were only slightly increased in the SF of RA patients with

respect to the plasma of the same patients, OPG levels were further increased ($P < 0.01$) in SF with respect to the plasma of RA patients (Fig. 1B). Of note, TNF-α was also significantly ($P < 0.05$) higher in SF with respect to the plasma of RA patients (Fig. 1B).

Immunohistochemical analysis of serial sections of synovial membrane samples obtained from RA patients revealed the presence of SC5b-9 deposits and OPG expression in the endothelium of vessels traversing the synovium, with a clear co-localization of SC5b-9 deposition and OPG-reactivity in the endothelium of small venulae capillaries (Fig. 2).

Clinically relevant concentrations of SC5b-9 and OPG promote PMN adhesion to endothelial cells

It is well known that neutrophilic infiltration is a key step in the onset and maintenance of inflammatory joint diseases [36]. Since the first event in the extravasation of leucocytes is represented by the firm adhesion to the endothelial layer, experiments were then designed to investigate whether SC5b-9 and OPG, used at the concentrations detected in the plasma and/or SF were able to promote PMN/endothelial cell interactions. HUVECs were grown to confluence and exposed to either SC5b-9 or OPG. As shown in Fig. 3, treatment with SC5b-9 as well as with recombinant OPG significantly increase the adhesion of PMNs to endothelial cells, suggesting that the concentrations of SC5b-9 and OPG found
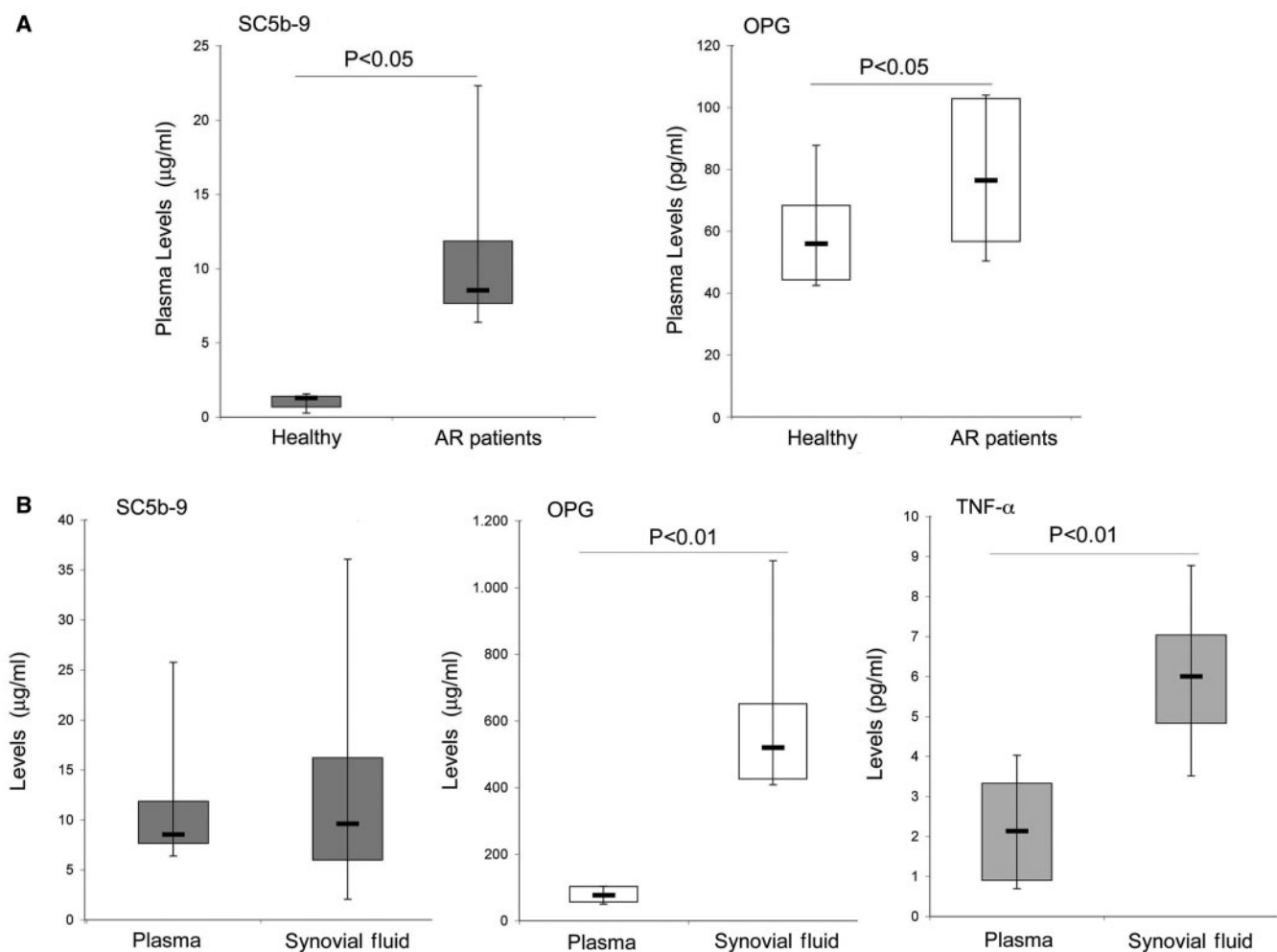

FIG. 1. High levels of SC5b-9 and OPG in RA patients. (**A**) Levels of SC5b-9 and OPG were determined by ELISA in plasma of RA patients and healthy control subjects. (**B**) Comparison between the plasma and SF levels of SC5b-9, OPG and TNF-α determined by ELISA in RA patients. Horizontal bars are median, upper and lower edges of box are 75th and 25th percentiles, lines extending from box are 10th and 90th percentiles.

in plasma and in SF (Fig. 1A and B) may efficiently promote leucocyte extravasation in the synovia of RA patients.

SC5b-9 up-regulates the expression and release of OPG by endothelial cells

To investigate the potential link between SC5b-9 and OPG, we next examined the ability of SC5b-9 to modulate the release of OPG by endothelial cells. As expected [35], untreated HUVECs released low levels of OPG in the culture supernatants,
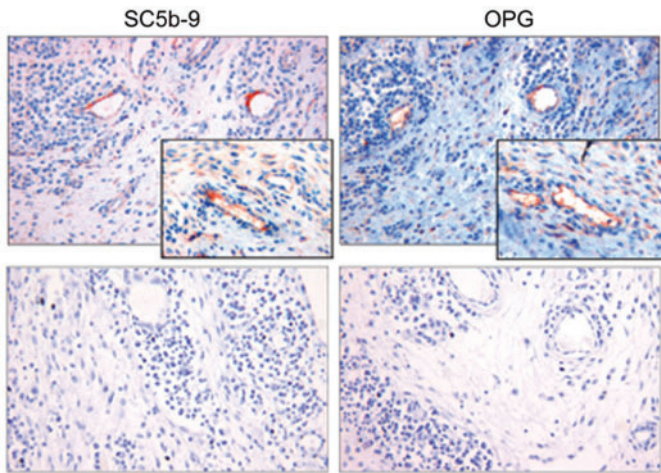

Fig. 2. SC5b-9 and OPG are co-localized on vascular endothelial cells in RA synovial membranes. Immunohistochemical evaluation of SC5b-9 and OPG staining on serial sections from synovial membrane samples. In the top panels, synovial membrane showed the presence of SC5b-9 deposition and intense OPG staining in the endothelium of vessels. In the bottom panels, negative controls of each immunohistochemical staining are shown. Original magnification, 200×; insets, 400×. Representative results of five separate experiments that gave similar results are shown.
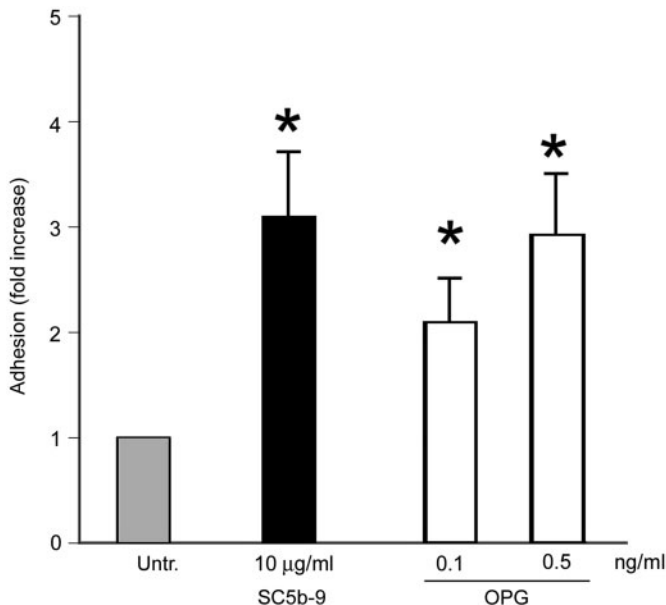

Fig. 3. Effect of SC5b-9 and OPG on PMN adhesion. HUVECs were either left untreated or exposed for 16 h to the indicated concentrations of SC5b-9 and OPG. After washing, PMNs were added to the endothelial monolayer, and the number of adhered cells was determined as described in Materials and methods section. Cell adherence on HUVECs is reported as fold of increase respect to cell adhesion in the absence of the indicated pre-treatment. Results are expressed as means ± S.D. of five experiments, each performed in triplicate. *$P < 0.05$.

while exposure to SC5b-9, used at concentrations in the range of those detected *in vivo*, time- and dose-dependently up-regulated OPG release (Fig. 4A and B). Of note, the simultaneous treatment of HUVECs with SC5b-9 plus the pro-inflammatory cytokine TNF-α, showed synergistic effects with respect to the production and release of OPG induced in response to SC5b-9 alone (Fig. 4C).

To ascertain whether SC5b-9 up-regulated OPG at the transcriptional level, we have generated a promoter–reporter construct containing the OPG promoter upstream of a luciferase reporter gene (pOPG-Luc). HUVECs were transfected with this construct, or with the empty control vector, and then were exposed to SC5b-9 for 18 h before assessing reporter gene activity. As shown in Fig. 5A, pOPG-Luc expression was significantly ($P < 0.05$) induced upon exposure of endothelial cells to SC5b-9. Taking into account that OPG is an NF-κB-dependent gene [37], we next evaluated the involvement of the NF-κB pathway in the up-regulation of OPG expression/release induced by SC5b-9.
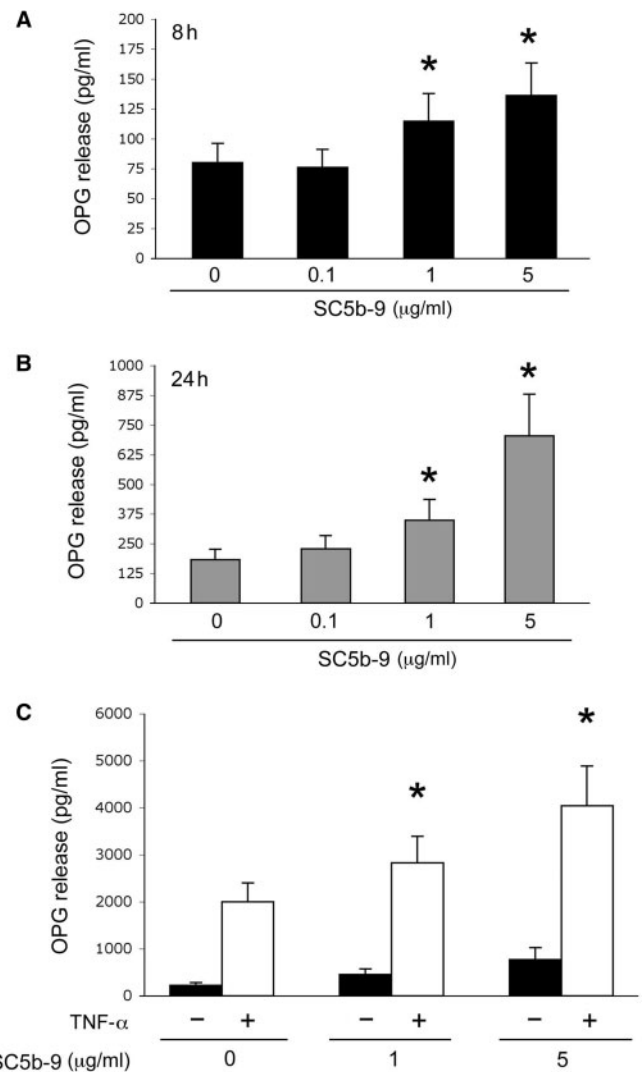

Fig. 4. SC5b-9 up-modulates OPG release in endothelial cells. HUVECs were left untreated or treated with the indicated concentrations of SC5b-9. OPG levels in culture supernatants were assessed by ELISA after 8 h (**A**) and 24 h (**B**) of treatment with SC5b-9 (0.1–5 µg/ml). *$P < 0.05$, compared with untreated. (**C**) OPG levels in culture supernatants were assessed by ELISA after 24 h of treatment with the indicated concentrations of SC5b-9 used either alone (black bars) or in combination with TNF-α (1 ng/ml; white bars). *$P < 0.05$, compared with SC5b-9. (**A–C**) Data are expressed as means ± S.D. of results from four independent experiments, each performed in duplicate.
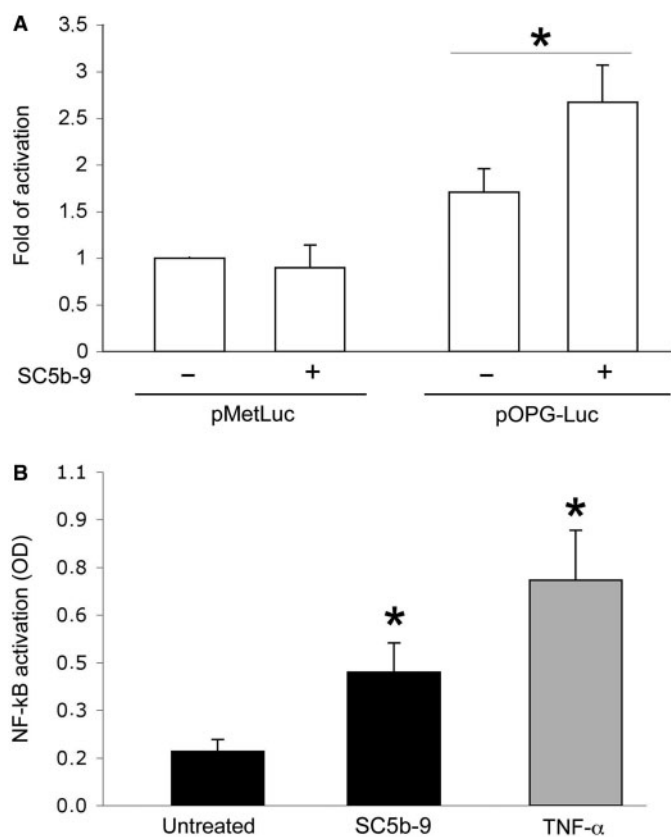
FIG. 5. SC5b-9 up-regulates OPG-promoter through activation of NF-κB. (**A**) HUVECs were transfected with pOPG-Luc or empty pMetLuc-Reporter vector. After recovery, transfected cells were stimulated as indicated and luciferase activity was assessed. pMetLuc was set as unit and results were calculated as fold of activation. Data are expressed as means ± S.D. of three independent experiments. *$P < 0.05$. (**B**) HUVECs were either left untreated or stimulated with SC5b-9 (5 µg/ml) for 30 min. NF-κB-p65 DNA binding activity was determined as absorbance values (OD) per microgram of cell lysate protein. NF-κB activation by TNF-α (10 ng/ml) is also shown for comparison. Results are expressed as means ± S.D. of three independent experiments, each performed in duplicate. *$P < 0.05$, compared with untreated cultures.

By using an oligonucleotide containing the kB consensus site (TransAM), a significant induction of p65/RelA NF-κB-DNA binding over untreated cultures was observed after 30 min of SC5b-9-treatment (Fig. 5B).

Discussion

Although the data concerning the levels of OPG in the sera of patients affected by RA are heterogeneous [20–26], a couple of studies have clearly shown that OPG concentrations were higher in patients with long-standing RA with respect to early RA, which in turn showed OPG levels more elevated than in controls [21, 26]. Consistently, we have observed significantly higher levels of OPG in plasma of active RA patients with respect to healthy controls. The same RA patients were also characterized by a significant increase of the plasmatic soluble terminal complement complex SC5b-9, compared with the healthy controls. Starting from these observations, we have demonstrated, by a series of *in vitro* experiments, that concentrations of SC5b-9 compatible with those measured in the plasma and/or SF of RA patients, significantly up-regulated the release of OPG in endothelial cultures. Moreover, we have demonstrated that SC5b-9 induced OPG expression in endothelial cells acting at the transcriptional level, likely through the NF-κB pathway. Although it has been shown that OPG is an NF-κB-dependent gene [37], and we have previously demonstrated that its expression and release in endothelial cells is

induced by the pro-inflammatory cytokine TNF-α [35], this is the first study demonstrating that also the complement cascade, and in particular SC5b-9, significantly induce OPG expression and release by endothelial cells. Furthermore, we have observed that TNF-α synergized with SC5b-9 in further promoting OPG release by endothelial cells, suggesting that different inflammatory stimuli cooperate in promoting the expression and release of OPG by endothelial cells. In this respect, it is noteworthy that while the concentrations of SC5b-9 were only slightly higher in SF than in plasma of RA patients, OPG concentrations were significantly ($P < 0.01$) greater in SF with respect to the plasma of the same RA patients. Of note, TNF-α was also significantly ($P < 0.05$) higher in SF with respect to the plasma of RA patients. Thus, it is possible to suppose a cooperation between SC5b-9 and TNF-α in SF, which results in the induction of the higher levels of OPG detected in SF *vs* plasma of RA patients. The relevance of our *in vitro* data was underscored by *in situ* immunohistochemical analysis of synovial lesions, which clearly showed a co-localization of SC5b-9 deposition and strong OPG expression in the endothelium, suggesting a potential link between these two molecules also *in vivo*. Moreover, the ability of both SC5b-9 and OPG to promote neutrophil–endothelial cell adhesion indicates that both molecules are actively involved in the tissue inflammation [36].

In conclusion, our data clearly indicate that OPG is a target gene of the complement-cascade and might contribute to the pro-inflammatory cascade characteristic of RA. Although different studies have originally focused the attention on beneficial effects of elevated OPG levels in counteracting the RANKL-mediated induction of osteoclastogenesis and thus preventing bone erosion in RA patients [22–25], our data point to the possibility that elevated OPG levels both in the plasma and in the SF might rather contribute to pathogenetic aspects of RA, such as increased adhesiveness of PMNs to endothelial cells. Moreover, beside RANKL, OPG is able to interact with a comparable affinity with TRAIL, and accumulating data indicate that the interplay among RANKL, OPG and TRAIL is more complex than originally thought [38–42]. In this respect, it is noteworthy that cultured rheumatoid fibroblast-like synovial cells are susceptible to TRAIL-mediated apoptosis, but recombinant OPG inhibits the pro-apoptotic activity of TRAIL [43]. Thus, it has been proposed that inflammatory cytokines might promote the growth of rheumatoid synovial tissues indirectly, through stimulation of OPG production and its interference with TRAIL-death signal [43]. It should also be mentioned that the pro-adhesive and pro-inflammatory activities of OPG [27, 28] might be totally unrelated to its ability to neutralize the activity of RANKL and TRAIL.

Rheumatology key messages

- SC5b-9 induces OPG expression and release by endothelial cells.
- SC5b-9-mediated up-regulation of OPG may be an important mechanism whereby complement contributes in promoting and/or enhancing the inflammation in RA.

Disclosure statement: The authors have declared no conflicts of interest.

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